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(54) A process for immobilizing an enzyme on an electrode surface.

(57) A process for immobilizing an enzyme on an electrode surface is provided. The process comprises steps of:

(a) electroplating the electrode surface, and  
(b) immobilizing thereon the enzyme and a mediator of electron transfer by means of an electropolymerizing substance.

The enzyme immobilized electrode thus obtained has a broader response range and a longer lifetime.

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## A PROCESS FOR IMMOBILIZING AN ENZYME ON AN ELECTRODE SURFACE

### BACKGROUND OF THE INVENTION

#### Field of the invention

The present invention relates to a biosensor, particularly a biosensor comprising an immobilized enzyme electrode.

#### Description of the prior art

A biosensor comprising an electrode on the surface of which an enzyme and a mediator of electron transfer are immobilized was described by Anthony E.G. Cass, et al. in "Analytical Chemistry" Vol. 56 (1984), No. 4, pages 667-671. This biosensor is one that was prepared by depositing, as a mediate of electron transfer,  $15\mu\text{L}$  of 1,1'-dimethylferrocene (0.1M in toluene) onto the surface of graphite electrodes (4 mm diameter), which had been heated at  $100^\circ\text{C}$  in air for 40 hours and allowed to cool; air-drying the deposited electrodes; immersing the dried electrodes into an aqueous solution of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide p-methyl-toluenesulfonate for 80 minutes at  $20^\circ\text{C}$ , and then into an acetate buffer solution (0.1M, pH 9.5) containing glucose oxidase (12.5 mg/mL) for 90 min at  $20^\circ\text{C}$ ; and covering the electrodes with a polycarbonate membrane.

A biosensor comprising an electrode on the surface of which an enzyme is immobilized by an electropolymerizing substance was described by Mirtha Umana, et al. in "Analytical Chemistry" Vol. 58 (1986), No. 14, pages 2979-2983. This biosensor is one that was prepared by potentiostatically electrolyzing an aqueous solution of KCl (0.1M), pyrrole (0.5M) and glucose oxidase (500 units/mL) by using platinum or glassy carbon working electrode and Ag/AgCl reference electrode at the potential 0.65 V.

A biosensor comprising an electrode on the surface of which a micro-platinized layer is deposited and then an enzyme is immobilized thereon was described by Yoshihito Ikariyama, et al. in "Analytical Letters" Vol. 20 (1987), No. 7, pages 1407-1416. This biosensor is one that was prepared by platinizing a platinum electrode (5-200  $\mu\text{m}$  diameter) in an aqueous solution of hexachloroplatinate (33 mg/mL) and lead acetate (0.6 mg/mL), by potentiostatic or galvanostatic electrolytic reduction; and then immersing the platinized

platinum electrode in a phosphate buffered solution containing glucose oxidase (1.5 mg, 110 unit/mg) for 10 minutes.

These known biosensors prepared by prior art enzyme immobilization methods however have problems of a short lifetime owing to leaching of the immobilized enzyme or mediator of electron transfer out of the electrode surface, and a narrow response range owing to insufficiency of absolute amounts of them as actually immobilized.

### SUMMARY OF THE INVENTION

An object of the present invention is therefore to present a process for immobilizing an enzyme on an electrode surface that enables to obtain a biosensor having a long lifetime and a wide response range, which biosensor has sufficient amounts of an enzyme and a mediator of electron transfer as actually immobilized on the electrode surface and which biosensor is capable of preventing leaching of them out of the electrode surface.

Thus the present invention provides a process for immobilizing an enzyme on an electrode surface comprising steps of:

(a) electropatenting the electrode surface, and  
 (b) immobilizing thereon the enzyme and a mediator of electron transfer by means of an electropolymerizing substance.

The process step (b) may be performed in the following single stage (b<sub>1</sub>) or in the following two stages (b<sub>2</sub>) and (b<sub>3</sub>):

(b<sub>1</sub>) forming on the electropatented electrode surface, a polymer coating layer essentially consisting of the enzyme, the mediator of electron transfer and the electropolymerizing substance, by electropolymerization, or

(b<sub>2</sub>) first, physically adsorbing the enzyme to the electropatented electrode surface, and

(b<sub>3</sub>) then, forming thereon a polymer coating layer essentially consisting of the mediator of electron transfer and the electropolymerizing substance, by electropolymerization.

Through said single stage (b<sub>1</sub>), obtainable is a biosensor comprising an immobilized enzyme electrode which has, on the electrode surface, a layer of an electropatented metal containing the enzyme and a mediator of electron transfer, and a layer of an electropolymerizing substance containing the enzyme and the mediator of electron transfer, in this order.

Through said two stages (b<sub>2</sub>) and (b<sub>3</sub>), obtainable is a biosensor comprising an immobilized en-

zyme electrode which has, on the electrode surface, a layer of an electroplated metal containing the enzyme, and a layer of an electropolymerized substance containing a mediator of electron transfer, in this order.

In order to obtain a uniform electroplated metal layer, it is advantageous to subject the electrode surface to an electrochemical pretreatment before the electroplating step (a).

The physical adsorption stage (b<sub>2</sub>) may be effectively performed when an electric potential is charged.

In view of solubility, the polymer coating layer formation stage (b<sub>1</sub>) or (b<sub>3</sub>) is performed preferably under substantially non-aqueous condition.

By electroplating a metal layer on the electrode surface, a large number of micro-pores are formed on the electrode surface; and the enzyme and the mediator for electron transfer are entrapped therein and covered and immobilized with the electropolymerizing substance.

In case wherein the electroplated layer and the electropolymerized layer both containing the enzyme and the mediator for electron transfer, it is possible to effectively perform the electron transfer reaction by the enzyme and so to obtain excellent linearity of sensor output current against enzyme concentration.

On the other hand, in case wherein the electroplated metal layer containing the enzyme and the electropolymerized layer containing the mediator of electron transfer are formed in this order, it is possible to obtain a long lifetime biosensor, since the enzyme is effectively protected by the electropolymerized layer.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a schematic illustration of the process steps of the present invention including two courses of (1) Figs. 1A, 1B and 1C and (2) Figs. 1A, 1B, 1D and 1E.

Fig. 2 shows a calibration line of glucose sensor obtained by the process steps of the Figs. 1A, 1B and 1C.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention will be explained below further in detail with respect to its some embodiments shown as working examples.

#### Example 1

A course of the process steps shown in Figs. 1A, 1B and 1C was taken.

An electrode 1 shown in Fig. 1A was prepared by sputtering titanium and gold on a sapphire substrate of 2.3 x 0.4 mm.

The electrode 1 was immersed in 0.1 M sulfuric acid solution and subjected to a potential sweeping treatment at a sweeping rate of 200 mV/sec for 10 minutes, at an electrode potential of -1.5 to +1.5 V against a saturated calomel electrode as a reference electrode.

The electrode 1 was then electroplated to form a platinum layer 2, as shown in Fig. 1B, by reduction in an aqueous solution of 33 mg/mL of chloroplatinic acid and 0.6 mg/mL of lead acetate at 100  $\mu$ A for one minute and at 200-225  $\mu$ A for ten minutes, using a platinum electrode as a counter electrode.

The platinized electrode 1 then was immersed in an acetonitrile solution of 20 mg/mL of glucose oxidase 3 shown in Fig. 1C, 10 mM of ferrocene carboxylic acid 4 shown in Fig. 1C, 0.1 M of tetraethylammonium-p-toluene sulfonic acid, 0.5 M of tetraethylammonium chloride and 0.2 M of pyrrole 5 shown in Fig. 1C and the pyrrole 5 was electropolymerized galvanostatically at electric current density of 3 mA/cm<sup>2</sup> to form a polymer coating layer thereby to immobilize the glucose oxidase 3 and the ferrocene carboxylic acid 4 on the platinized surface of the electrode 1.

#### Example 2

A course of the process steps shown in Figs. 1A, 1B, 1D and 1E was taken.

The procedures up to electroplating of electrode corresponding to Figs. 1A and 1B were the same as those of Example 1.

Then the platinized electrode 1 was immersed in a phosphate buffered solution of pH 7.0 containing 20 mg/mL glucose oxidase 3 shown in Fig. 1D at 4 °C for 12 hours to take up the glucose oxidase 3 into the electroplated platinum layer 2. It is possible to control the amount of the glucose oxidase 3 to be taken up into the electroplated platinum layer 2 by charging an electric potential to the electrode 1.

Next, the electrode 1 was immersed in an acetonitrile solution of 10 mM of ferrocene carboxylic acid 4 shown in Fig. 1D, 0.1 M of tetraethylammonium-p-toluene sulfonic acid, 0.5 M of tetraethylammonium chloride and 0.2 M of pyrrole 5 shown in Fig. 1D and the pyrrole 5 was electropolymerized galvanostatically at electric current density of 3 mA/cm<sup>2</sup> to form a polymer coating layer thereby to immobilize the glucose oxidase 3 and the ferrocene carboxylic acid 4 on the platinized surface of the electrode 1.

ed surface of the electrode 1.

In Fig. 2, a calibration line of the glucose sensor produced in Example 1 is shown. It is seen that straight linearity extends up to 50 mM glucose concentration. This means that the range of linearity has been broadened 2.5 to 5 times of prior art glucose sensors.

In the above Examples, glucose oxidase 3 was used as the enzyme, ferrocene carboxylic acid 4 as the mediator for electron transfer and pyrrole 5 as the electropolymerizing substance. It is also possible however to use any oxidase series enzymes containing a flavin adenine dinucleotide (FAD), such as D-amino-acid oxidase, pyruvate oxidase, choline oxidase, taking advantage of the fact that these enzymes can easily be used for electron transfer reactions owing to FAD. It is also possible to replace the ferrocene carboxylic acid by any other ferrocene derivatives such as 1,1'-dimethyl ferrocene, methyl ferrocene, hydroxymethyl ferrocene. The reason for using ferrocene derivatives is that they facilitate the electron transfer from the enzyme. Further, the pyrrole may be replaced by any other electropolymerizable substances such as N-methylpyrrole, aniline.

As for the solvent for electropolymerization, acetonitrile was used in the above Examples. Other organic solvents, such as nitromethane, N,N-dimethylformamide, N,N-dimethylacetamide, N,N-diethylacetamide, N-methylformamide, tetrahydrofuran, formamide or dimethylsulfoxide may also be used, since these are good solvents also for the mediator for electron transfer. Water may also be used, preferably together with a small amount of a surface active agent, but many mediators for electron transfer are difficultly soluble in water.

As explained in detail as above, the present invention has enabled to immobilize sufficient amounts of an enzyme and a mediator of electron transfer, by electroplating the electrode surface to form many micro-pores and by covering the outermost layer of the electrode with an electropolymerized substance. Accordingly, the response range of the sensor has been broadened 2.5 to 5 times and the lifetime of the sensor has been prolonged.

## Claims

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1. A process for immobilizing an enzyme on an electrode surface comprising steps of:

(a) electroplating the electrode surface, and

(b) immobilizing thereon the enzyme and a mediator of electron transfer by means of an electropolymerizing substance.

2. The process according to claim 1, wherein

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the immobilizing step (b) comprises a single stage of:

(b<sub>1</sub>) forming on the electroplated electrode surface, a polymer coating layer essentially consisting of the enzyme, the mediator of electron transfer and the electropolymerizing substance, by electropolymerization.

3. The process according to claim 1, wherein the immobilizing step (b) comprises two stages of:

(b<sub>2</sub>) first, physically adsorbing the enzyme to the electroplated electrode surface, and

(b<sub>3</sub>) then, forming thereon a polymer coating layer essentially consisting of the mediator of electron transfer and the electropolymerizing substance, by electropolymerization.

4. The process according to claim 1, wherein the electrode surface is subjected to an electrochemical pretreatment before the electroplating step (a).

5. The process according to claim 2, wherein the physical adsorption stage (b<sub>2</sub>) is performed by charging electric potential.

6. The process according to claim 2 or 3, wherein the polymer coating layer formation stage (b<sub>1</sub>) or (b<sub>3</sub>) is performed under substantially non-aqueous condition.

7. A biosensor comprising an immobilized enzyme electrode which has, on the electrode surface, a layer of an electroplated metal containing the enzyme and a mediator of electron transfer, and a layer of an electropolymerizing substance containing the enzyme and the mediator of electron transfer, in this order.

8. A biosensor comprising an immobilized enzyme electrode which has, on the electrode surface, a layer of an electroplated metal containing the enzyme, and a layer of an electropolymerized substance containing a mediator of electron transfer, in this order.

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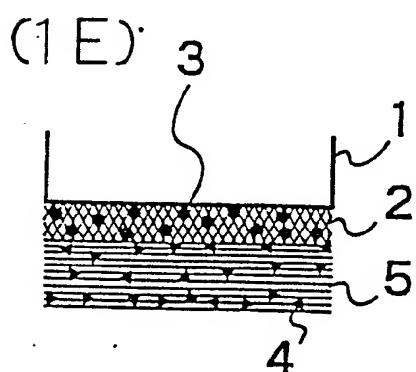
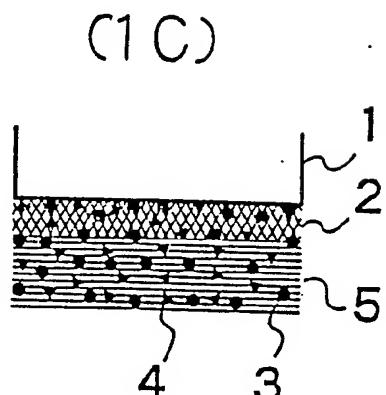
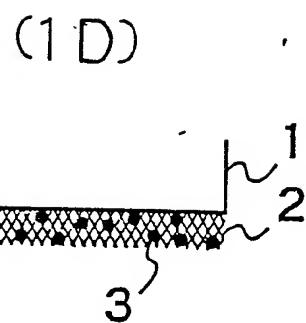
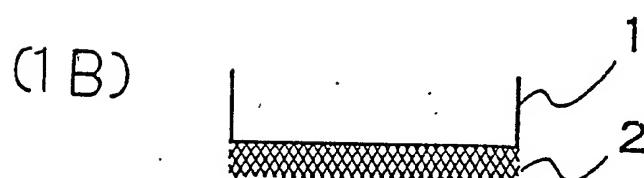
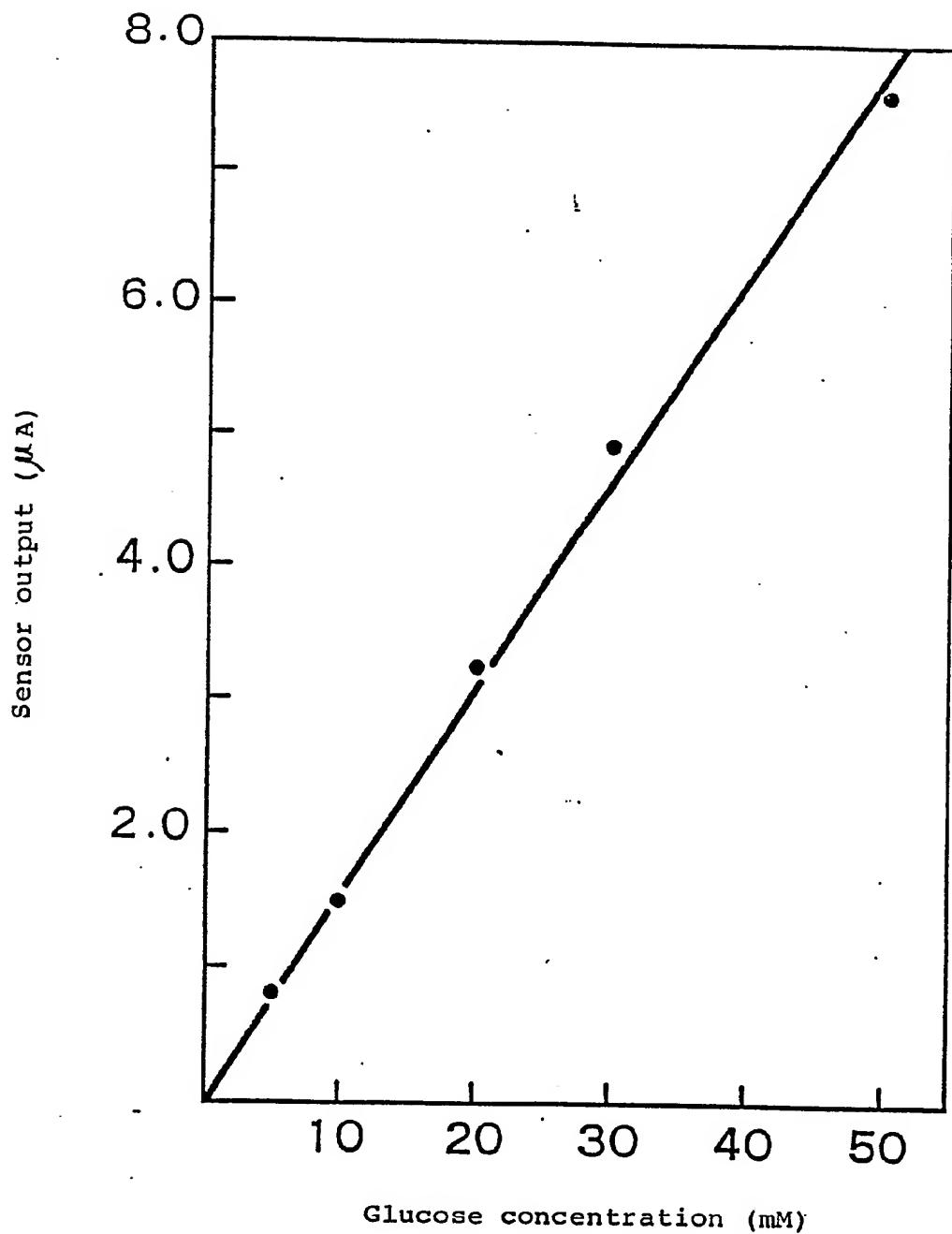


FIG. 2





EP 89 12 0516

DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Category	Citation of document with indication, where appropriate, of relevant passages		
Y	JOURNAL OF THE CHEMICAL SOCIETY, CHEMICAL COMMUNICATIONS, no. 15, 1st August 1988, pages 1019-1020, London, GB; C. IWAKURA et al.: "Simultaneous immobilization of glucose oxidase and a mediator in conducting polymer films" * Whole document * ---	1-8	C 12 M 1/40
Y	EP-A-0 304 494 (JAPAN) * Page 24, line 4 - page 26, line 10; claims 1-8 * ---	1-8	
A	CHEMICAL ABSTRACTS, vol. 109, 1988, page 410, abstract no. 225991t, Columbus, Ohio, US; Y. IKARIYAMA et al: "High performance micro-enzyme sensor using platinized microelectrode", & BULL. CHEM. SOC. JPN. 1988, 61(10), 3525-30 * Abstract * ---	1,3,7,8	
A,D	CHEMICAL ABSTRACTS, vol. 108, 1988, page 296, abstract no. 18602f, Columbus, Ohio, US; Y. IKARIYAMA et al: "Micro-enzyme electrode prepared on platinized platinum", & ANAL. LETT. 1987, 20(9), 1407-16 * Abstract * ---	1,3,7,8	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C 12 M C 12 N
A,D	CHEMICAL ABSTRACTS, vol. 105, 1986, abstract no. 183095p, Columbus, Ohio, US; M. UMAN et al.: "Protein-modified electrodes. The glucose oxidase/polypyrrole system" * Abstract * -----	1-3,7,8	
The present search report has been drawn up for all claims			
Place of search THE HAGUE	Date of completion of the search 20-12-1989	Examiner EPAILLARD P.J.H.M.	
CATEGORY OF CITED DOCUMENTS		<p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ..... &amp; : member of the same patent family, corresponding document</p>	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			